

## **METHODS OF REGULATING DIFFERENTIATION IN STEM CELLS**

### **Cross-Reference to Related application**

This application is a continuation of International Application No.

5 PCT/AU03/00713, filed .

### **Technical Field**

The present invention relates to methods for inhibiting spontaneous differentiation of stem cells. The invention also relates to media useful in propagating stem cells in an undifferentiated state, methods for identifying agents useful for inhibiting stem cell differentiation, and methods of treating stem cell related disorders.

### **Background Art**

15 In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ, cell type or tissue type or, at least potentially, into a complete embryo. ES cells may be derived from the inner cell mass of the blastocyst, which have the ability to differentiate into tissues representative of the three embryonic germ layers (mesoderm, ectoderm, endoderm), and into the extra-embryonic tissues that support development.

25 Human embryonic stem cells (hES cells) are pluripotent cell lines derived from the inner cell mass of the blastocyst. These cells have the ability to differentiate into functional tissues representative of the three embryonic germ layers (mesoderm, ectoderm, endoderm), and into extra-embryonic tissues that support development. Because of their ability to generate these different cellular fates, hES cells are considered to be of great potential for future therapies.

However, during routine culture *in vitro*, established hES cell lines have a tendency to spontaneously differentiate. Because the pluripotency of

these cells is associated with their undifferentiated state, it is desirable to find a way to limit this spontaneous differentiation. Contrary to what is seen in mouse embryonic stem cells, leukemia inhibitory factor (LIF) does not prevent the spontaneous differentiation of hES cells [1]. Thus, a common way to grow and then to maintain hES cells in an optimum state is to cultivate them on feeder layers, which are constituted by primary mouse embryonic fibroblasts (MEF), in media supplemented with high doses of foetal calf serum.

However, serum contains a wide variety of biologically active compounds that might have the potential to adversely affect hES cell growth and differentiation. Furthermore, there is a biosafety issue if cells cultured in animal serum are subsequently used for implantation in a human or for the production of a biological therapeutic.

With regard to these issues and in order to establish a serum-free culture system to grow hES cells, it is of great importance to identify the specific factors in serum that are responsible for its beneficial effect on the growth of hES cells. Thus, alternative approaches to traditional culture systems are desirable, such as the use of a serum replacement medium such as Knockout Serum Replacement [2, 3].

Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are two small bioactive lysophospholipids, present in serum (at concentration of up to 1 and 5  $\mu$ M respectively) [4], released by activated platelets, which act on a wide range of cell types derived from the three developmental germ layers. Most of the effects of these lysophospholipids seem to be mediated by specific lysophospholipid G-protein coupled receptors (LPL receptors) previously named endothelial differentiation gene (Edg) receptors.

Up to now, eight distinct mammalian LPL/Edg receptors have been identified: S1P<sub>1</sub>/Edg-1, S1P<sub>2</sub>/Edg-5, S1P<sub>3</sub>/Edg-3, S1P<sub>4</sub>/Edg-6 and S1P<sub>5</sub>/Edg-8 are specific for S1P while LPA<sub>1</sub>/Edg-2, LPA<sub>2</sub>/Edg-4 and LPA<sub>3</sub>/Edg-7 are specific for LPA (for reviews see [5, 6]). Each of these receptors is coupled to at least one G protein and can activate or inhibit specific signalling pathways. For instance, all these receptors are coupled to G<sub>i/o</sub> proteins (for review see [5, 6]).

By activating notably these  $G_{i/o}$  proteins, S1P and LPA can stimulate the extracellular-signal-regulated kinases 1 and 2 (ERK1/2), which are members of the mitogen-activated protein (MAP) kinase family, and thus are involved in regulation of major cellular events, such as cell proliferation or  
 5 differentiation. S1P and LPA are potent biological agents involved in numerous cell events, such as proliferation, differentiation, death or migration (for review see [5]) since the very early stages of development.

S1P stimulates mammalian angiogenesis, at least via  $S1P_1$  and  $S1P_2$  [7-10]. Thus,  $S1P_1$  knockout mice show impaired blood vessel maturation.  
 10 Moreover, in the zebrafish, S1P is required for normal heart development [11]. Thus, in these animals, the mutation of the gene *mil* that encodes the S1P receptor Mil (very similar to the mammalian  $S1P_2$  receptor) impairs migration of cardiac progenitor cells [11].

On the other hand, LPA seems to be mainly involved in neurogenesis  
 15 [12]. For instance, LPA, probably via  $LPA_1$ , stimulates cell cycle-morphological changes and cell migration of cultured cortical neuroblasts. Moreover, LPA, probably via  $LPA_2$ , regulates the migration of post-mitotic neurons to their final destination. Last but not least,  $LPA_1$  knockout mice present abnormal cerebral cortices and olfactory bulbs, probably due to  
 20 impaired development, demonstrating  $LPA_1$  is essential for a normal brain development [13].

Within serum, Platelet-Derived Growth Factor (PDGF) is a major protein growth factor that has been widely described as a potent mitogen of numerous kinds of cells. PDGF has also been shown to induce chemotaxis,  
 25 actin re-organization, and to prevent apoptosis. This growth factor belongs to a family of dimeric isoforms of polypeptide chains, A, B, C and D that act through different tyrosine kinase receptors: PDGFR- $\alpha$  and PDGFR- $\beta$ .

S1P and PDGF have additional effects that induce biological responses. Thus S1P and PDGF are able to regulate smooth muscle cell  
 30 migration, proliferation and vascular maturation. Moreover, Hobson *et al.* (2001), and Rosenfeld *et al.* (2001) demonstrated that PDGF-stimulated cell motility is  $S1P_1$ -dependent in HEK 293 cells and MEF [14, 15] while Kluk *et al.* (2003) showed that this effect was independent of  $S1P_1$  in vascular

smooth muscles and MEF [16]. Last but not least, it is now proposed that PDGF is able to stimulate the enzyme sphingosine kinase, which leads to an increase in S1P intracellular concentration [17], an effect that would be responsible for PDGF-induced proliferation in Swiss 3T3 cells [17] and  
5 vascular smooth muscle cells [18].

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common  
10 general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

### **Summary of the Invention**

In one aspect the present invention provides a method for modulating  
15 spontaneous differentiation of a stem cell, which method comprises incubating the stem cell in the presence of an agonist of a LPL receptor and/or a ligand of a class III tyrosine kinase receptor.

In another aspect the present invention provides a serum free or substantially serum free medium useful for modulating spontaneous  
20 differentiation of a stem cell, comprising an agonist of a LPL receptor and/or a ligand of a class III tyrosine kinase receptor.

Another aspect of the present invention provides a method of treating or preventing a disorder of stem cell differentiation comprising administering to an animal in need thereof a composition containing an agonist of a LPL  
25 receptor and/or a ligand of a class III tyrosine kinase receptor.

Another aspect of the present invention provides a pharmaceutical composition comprising a class III tyrosine kinase receptor ligand and/or a LPL receptor agonist.

In a further aspect the present invention provides a method of  
30 producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor and/or a ligand of a class III tyrosine kinase receptor

In another aspect the present invention provides a method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor and/or a ligand of a class III tyrosine kinase receptor.

### **Description of the Invention**

The present inventors investigated the role of the LPL receptor agonists S1P, dihydro S1P and LPA, and the ligand of a class III tyrosine kinase receptor, PDGF, in modulating the fate of hES cells in culture.

The present inventors have established that hES cells are target cells for S1P, dihydro S1P, LPA and PDGF, through expression of the LPL receptors, PDGFR- $\alpha$  and PDGFR- $\beta$  and through stimulation of ERKs by these agonists. Moreover the present inventors have found that S1P and PDGF slightly inhibit the spontaneous differentiation of hES cells while co-incubation with both S1P and PDGF strongly reduces the spontaneous differentiation of hES cells. These findings provide a basis for the establishment of a serum-free culture medium for stem cells and in particular hES cells.

Throughout the description and claims of this specification, the word "comprise" and variations of that word, such as "comprising" and "comprises" are not intended to exclude other additives, steps or integers.

In a first aspect the present invention provides a method for modulating spontaneous differentiation of a stem cell, which method comprises incubating the stem cell in the presence of an agonist of a LPL receptor.

In a second aspect the present invention provides a method for modulating spontaneous differentiation of a stem cell, which method comprises incubating the stem cell in the presence of a ligand of a class III tyrosine kinase receptor.

In a third aspect the present invention provides a method for modulating spontaneous differentiation of a stem cell, which method

comprises incubating the stem cell in the presence of an agonist of a LPL receptor and a ligand of a class III tyrosine kinase receptor.

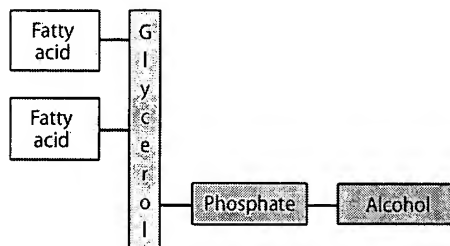
Sphingosine-1-phosphate (S1P), an agonist of the LPL receptors has the ability to at least partially inhibit the spontaneous loss of stem cell phenotype in cell culture. It has also been found that the method does not affect the ability of stem cells to proliferate.

Preferably, the LPL receptor is selected from the group consisting of S1P1, S1P2 and S1P3.

As used herein the term "modulating the differentiation of a stem cell" includes the inhibition or enhancement of cellular differentiation. The term also includes partial inhibition or enhancement of cellular differentiation. In a preferred form of the method, the modulation is inhibition of differentiation.

Typically the agonist is a phospholipid.

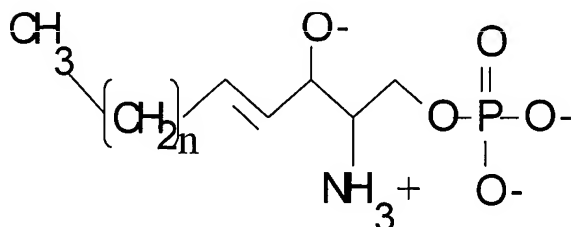
As used herein, the term "phospholipid" refers to a molecule that includes a backbone attached to two fatty acid moieties and a phosphate group. The backbone on which the fatty acid molecules are attached is variable and may be based on glycerol or sphingosine for example. A diagram of a generic phospholipid is shown below.



The term "lysophospholipid" refers to a phospholipid molecule where one of the fatty acid chains has been removed. The removal of a fatty acid chain may be accomplished by treatment of the phospholipid with an enzyme such as phospholipidase A2.

The phospholipid or lysophospholipid may have a sphingosine backbone, and particularly, the lysophospholipid may be a phosphorylated amino alcohol. Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC or functional equivalents thereof.

In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate (S1P) or a functional equivalent thereof. S1P is a



The skilled person will understand that bioactive molecules such as phospholipids and lysophospholipids may be altered in a number of ways and still retain biological activity. Accordingly, the scope of the present invention includes altered forms of phospholipids and lysophospholipids that retain their LPL receptor agonist activity. The scope of the present invention also includes synthetic peptidic agonists of the LPL receptors.

The skilled person will be familiar with methods which can be applied to testing phospholipids or lysophospholipids for the ability to modulate the ability of a stem cell to differentiate. Suitable methods are found herein, and include reactivity with antibodies such as GCTM-2 which are directed to stem cell specific markers, and simple morphological evaluation of cells by light microscopy.

For example, the effect of the agonist on the differentiation of stem  
20 cells into neuronal or endodermal lineages may be studied by analysis of  
marker expression as shown in PCT/AU01/00278 and PCT/AU01/00735.

The phospholipid or lysophospholipid may be extracted from a biological source such as serum. In addition, mast cells and monocytes are able to produce S1P while adipocytes produce LPA, however the main source of LPA and S1P is activated platelets. Alternatively, the phospholipid may be synthesised by procedures well known in the field of organic chemistry.

Preferably, cells that have been exposed to a LPL receptor agonist are not substantially negatively affected in their ability to proliferate. Therefore, 30 an advantage of the methods and compositions described herein is that it is

possible to expand a population of hES cells without leading to a loss in pluripotency. Methods for determining the proliferative capability of a hES cell will be known by the skilled person and include detection of the cell proliferation marker PCNA as described herein.

5 Typically the ligand is a PDGF or functional equivalent thereof.

The tyrosine kinase receptor may be PDGFR- $\alpha$  or PDGFR- $\beta$ .

In a preferred embodiment the PDGF is PDGF $\alpha\alpha$ , PDGF $\alpha\beta$  or PDGF $\beta\beta$  which bind to the two types of receptors.

10 The method may also include use of TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that have additive or synergistic effects with S1P in other cell types.

The stem cell may be derived from foetal tissue or adult tissue.

15 The stem cell is typically an ES cell. Preferably the stem cell is a hES cell. As used herein the term "embryonic stem cell" means a cultured cell line derived from preimplantation stages of development capable of differentiation into tissues representative of all three embryonic germ layers.

These cells:

- 20 - express SSEA-3, SSEA-4, TRA 1-60, GCTM-2, alkaline phosphatase and Oct-4
- Grow as flat colonies with distinct cell borders
- Differentiate into derivatives of all three embryonic germ layers
- Are feeder cell dependent (feeder cell effect on growth not reconstituted by conditioned medium from feeder cells or by
- 25 feeder cell extracellular matrix)
- Are highly sensitive to dissociation to single cells and show poor cloning efficiency even on a feeder cell layer
- Do not respond to Leukemia Inhibitory Factor

30 In a fourth aspect the present invention provides a serum free medium useful for modulating spontaneous differentiation of a stem cell having a LPL receptor, comprising an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor.



In a fifth aspect the present invention provides a serum free medium useful for modulating spontaneous differentiation of a stem cell, comprising a ligand of a class III tyrosine kinase receptor.

The medium is useful in propagating stem cells such as human  
5 embryonic stem cells in an undifferentiated state.

Typically the ligand is a PDGF or functional equivalent thereof.

The tyrosine kinase receptor may be PDGFR- $\alpha$  or PDGFR- $\beta$ .

In a preferred embodiment the PDGF is PDGF $\alpha\alpha$ , PDGF $\alpha\beta$  or PDGF $\beta\beta$ .

10 The medium may also include TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that have additive or synergistic effects with S1P.

Typically the agonist is a phospholipid.

Preferably the agonist is selected from the group consisting of S1P,  
15 LPA, PAF, dihydro S1P and SPC or functional equivalents thereof.

The stem cells may be derived from foetal tissue or adult tissue.

The stem cells are typically embryonic stem cells.

Preferably the stem cells are from embryonic tissue.

Typically the stem cells are of human origin.

20 The base medium is typically a standard serum free medium that is supplemented with phospholipids and ligand as well as a buffering agent. A suitable buffering agent is 25mM Hepes.

The medium is of use in inhibiting the differentiation of pluripotent stem cells.

25 The cell culture medium may be based on any of the base media known in the art useful for the growth and/or maintenance of stem cells such as hES cells. Such media include but are not limited to Dulbecco's Modified Eagles Medium (DMEM), KNOCKOUT-DMEM or hES medium. In a preferred form of the invention the medium is based on DMEM supplemented  
30 with insulin, transferrin and selenium.

The optimal concentration of LPL agonist in the medium may be determined by routine experimentation. However, in a preferred form of the invention the agonist is present in the medium at a concentration of from 0.1

μM to 10μM where the agonist is S1P. In a highly preferred form of the invention the agonist is present in the medium at a concentration of about 10μM. It would be expected that the optimum concentration will vary according to a number of parameters including the species of agonist, the line of stem cells being cultured, the base medium used, and other culture conditions such as temperature, carbon dioxide concentration, and humidity.

The optimal concentration of ligand in the medium may be determined by routine experimentation. However, in a preferred form of the invention the ligand is present in the medium at a concentration of from 1 ng/ml to 20ng/ml where the ligand is PDGFaa, PDGFab or PDGFbb. In a highly preferred form of the invention the ligand is present in the medium at a concentration of 20 ng/ml. Again, it would be expected that the optimum concentration will vary according to a number of parameters including the species of agonist, the line of stem cells being cultured, the base medium used, and other culture conditions such as temperature, carbon dioxide concentration, and humidity.

The skilled person understands that it is often necessary to culture hES cells on feeder cells, and the present invention contemplates methods including the use of such feeder cells. The concentration of agonist may also need to be optimised according to the feeder cell line used.

In a fifth aspect the present invention provides a stem cell grown and/or maintained in a cell culture medium of the invention.

Cells of the present invention will find many uses in biology and medicine. The properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide ranging potential applications of ES cell technology in human medicine-basic embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation are based on the assumption that it will be possible

to increase the proliferation and therefore grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation.

The present invention provides a method of producing a population of proliferating undifferentiated stem cells from a stem cell which method  
5 comprises incubating the stem cell in the presence of an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor.

The present invention also provides a method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of a ligand of a  
10 class III tyrosine kinase receptor.

The present invention further provides a method of producing a population of proliferating undifferentiated stem cells from a stem cell which method comprises incubating the stem cell in the presence of an agonist of the LPL receptor.

15 These methods therefore provide for the expansion of stem cell populations.

The invention also provides a population of undifferentiated stem cells produced by at least one of these methods.

Preferably, the LPL receptor is selected from the group consisting of  
20 S1P1, S1P2 and S1P3.

Typically the agonist is a phospholipid.

Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC or functional equivalents thereof.

In a highly preferred form of the invention the lysophospholipid is  
25 sphingosine-1-phosphate (S1P) or a functional equivalent thereof.

Typically the ligand is a PDGF or functional equivalent thereof.

The tyrosine kinase receptor may be PDGFR- $\alpha$  or PDGFR- $\beta$ .

In a preferred embodiment the PDGF is PDGF $\alpha\alpha$ , PDGF $\alpha\beta$  or PDGF $\beta\beta$  which bind to the two types of receptors.

30 The ligand may also be TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters.

The stem cell may be derived from foetal tissue or adult tissue.

The stem cell is typically an ES cell. Preferably the stem cell is a hES cell.

Another aspect of the present invention is a method of treating or preventing a disorder of stem cell differentiation comprising administering to  
5 an animal in need thereof a composition containing an agonist of a LPL receptor. Methods for the preparation of pharmaceutical compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 18<sup>th</sup> Edition, Mack Publishing Company, Easton, Pennsylvania, USA, the contents of which is incorporated herein.

10 The present invention also provides a method of treating or preventing a disorder of stem cell differentiation comprising administering to an animal in need thereof a composition containing an agonist of a LPL receptor.

The present invention also provides a method of treating or preventing a disorder of stem cell differentiation comprising administering to an animal in  
15 need thereof a composition containing a ligand of a class III tyrosine kinase receptor.

Another aspect of the present invention is a method of treating or preventing a disorder of stem cell differentiation comprising administering to an animal in need thereof a composition containing an agonist of a LPL  
20 receptor and a ligand of a class III tyrosine kinase receptor.

The present invention also provides a method of treating or preventing a disorder of stem cell differentiation comprising administering a stem cell as described herein. Disorders of stem cell differentiation are well known to those skilled in the art, and include, but are not limited to the following:

25

#### Acute Leukemias

Acute Lymphoblastic Leukemia (ALL)  
Acute Myelogenous Leukemia (AML)  
Acute Biphenotypic Leukemia  
Acute Undifferentiated Leukemia

#### Chronic Leukemias

Chronic Myelogenous Leukemia (CML)  
Chronic Lymphocytic Leukemia (CLL)

Juvenile Chronic Myelogenous Leukemia (JCML)  
 Juvenile Myelomonocytic Leukemia (JMML)

#### Myelodysplastic Syndromes

Refractory Anemia (RA)  
 Refractory Anemia with Ringed Sideroblasts (RARS)  
 Refractory Anemia with Excess Blasts (RAEB)  
 Refractory Anemia with Excess Blasts in Transformation (RAEB-T)  
 Chronic Myelomonocytic Leukemia (CMML)

#### Stem Cell Disorders

Aplastic Anemia (Severe)  
 Fanconi Anemia  
 Paroxysmal Nocturnal Hemoglobinuria (PNH)  
 Pure Red Cell Aplasia

#### Myeloproliferative Disorders

Acute Myelofibrosis  
 Agnogenic Myeloid Metaplasia (myelofibrosis)  
 Polycythemia Vera  
 Essential Thrombocythemia

#### Lymphoproliferative Disorders

Non-Hodgkin's Lymphoma  
 Hodgkin's Disease

#### Phagocyte Disorders

Chediak-Higashi Syndrome  
 Chronic Granulomatous Disease  
 Neutrophil Actin Deficiency  
 Reticular Dysgenesis

#### Inherited Metabolic Disorders

Mucopolysaccharidoses (MPS)  
 Hurler's Syndrome (MPS-IH)  
 Scheie Syndrome (MPS-IS)  
 Hunter's Syndrome (MPS-II)  
 Sanfilippo Syndrome (MPS-III)  
 Morquio Syndrome (MPS-IV)  
 Maroteaux-Lamy Syndrome (MPS-VI)  
 Sly Syndrome, Beta-Glucuronidase Deficiency (MPS-VII)  
 Adrenoleukodystrophy  
 Mucopolipidosis II (I-cell Disease)

Krabbe Disease  
 Gaucher's Disease  
 Niemann-Pick Disease  
 Wolman Disease  
 Metachromatic Leukodystrophy

#### Histiocytic Disorders

Familial Erythrophagocytic Lymphohistiocytosis  
 Histiocytosis-X  
 Hemophagocytosis

#### Inherited Erythrocyte Abnormalities

Beta Thalassemia Major  
 Sickle Cell Disease

#### Inherited Immune System Disorders

Ataxia-Telangiectasia  
 Kostmann Syndrome  
 Leukocyte Adhesion Deficiency  
 DiGeorge Syndrome  
 Bare Lymphocyte Syndrome  
 Omenn's Syndrome  
 Severe Combined Immunodeficiency (SCID)  
 SCID with Adenosine Deaminase Deficiency  
 Absence of T & B Cells SCID  
 Absence of T Cells, Normal B Cell SCID  
 Common Variable Immunodeficiency  
 Wiskott-Aldrich Syndrome  
 X-Linked Lymphoproliferative Disorder

#### Other Inherited Disorders

Lesch-Nyhan Syndrome  
 Cartilage-Hair Hypoplasia  
 Glanzmann Thrombasthenia  
 Osteopetrosis

#### Inherited Platelet Abnormalities

Amegakaryocytosis / Congenital Thrombocytopenia

#### Plasma Cell Disorders

Multiple Myeloma  
 Plasma Cell Leukemia  
 Waldenstrom's Macroglobulinemia

### Other Malignancies

Breast Cancer  
Ewing Sarcoma  
Neuroblastoma  
Renal Cell Carcinoma

Thus, the present invention may be used to treat a patient having a stem cell related disease by administration of a composition described herein, or by administering populations of stem cells produced by a method  
5 described herein

The agonist is typically a phospholipid. The phospholipid may be a lysophospholipid and may have a sphingosine backbone. Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC or functional equivalents thereof. S1P and dihydro S1P are  
10 lysophospholipids with a sphingosine backbone, as is SPC, while LPA is a lysophospholipid with a glycerol backbone, and PAF is a phospholipid with a glycerol backbone.

The tyrosine kinase receptor may be PDGFR- $\alpha$  or PDGFR- $\beta$  and the ligand a PDGF or functional equivalent thereof.

15 In a preferred embodiment the PDGF is PDGF $\alpha\alpha$ , PDGF $\alpha\beta$  or PDGF $\beta\beta$ .

The method may also include use of TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds that have additive or synergistic effects with S1P in  
20 other cell types.

Also provided is a pharmaceutical composition comprising a class III tyrosine kinase receptor ligand and a LPL receptor agonist. The composition may also include use of TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which again are compounds  
25 that have additive or synergistic effects with S1P in other cell types.

A skilled person will be able to provide formulations and dosage forms of the agonist. Furthermore, the optimum dosage for a given clinical situation could be determined by routine experimentation.

The compositions may be administered parenterally. For parenteral administration, the agonist and/or ligand may be combined with sterile aqueous or organic media to form injectable solutions or suspensions. The injectable solutions prepared in this manner may then be administered  
5 intravenously, intraperitoneally, subcutaneously, or intramuscularly. Additional methods of administration may include, but are not limited to, topical, sublingual, anal and vaginal methods of administration according to methods which are commonly known by those skilled in the art.

The amount of agonists or ligand used for preparation of a  
10 pharmaceutical composition should be varied according to principles well known in the art taking into account the severity of the condition being treated and the route of administration. In general, such a pharmaceutical composition would be administered to a warm blooded animal, preferably a mammal and most preferably a human, so that an effective dose, usually a  
15 daily dose administered in unitary or divided portions, is received. Dosages depend upon a number of factors, including the condition or disease being treated, characteristics of the subject and the type of pharmaceutical form or formulation used. Such deviations are within the scope of this invention.

Suitable pharmaceutically acceptable carriers for preparing a  
20 pharmaceutical composition include inert solid fillers or diluents and sterile aqueous or organic solutions. The antagonist and/or ligand are present in such pharmaceutical compositions in amounts sufficient to provide the desired dosage according to the range described above. Thus, for oral administration the agonist and/or ligand may be combined with a suitable  
25 solid or liquid carrier or diluent to form capsules, tablets, powders, syrups, solutions, suspensions and the like. The pharmaceutical compositions may, if desired, contain additional components such as flavorants, sweeteners, excipients and the like. Controlled release, sustained release, and delayed release oral or parenteral compositions may be used.

30 The tablets, pills, capsules, and the like may also contain one or more binders such as gum tragacanth, acacia, corn starch or gelatin; one or more excipients such as dicalcium phosphate; one or more disintegrating agents such as corn starch, potato starch, alginic acid; one or more lubricants such



as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin. When a dosage unit form is a capsule, for example a gel capsule, it may contain, in addition to or instead of materials of the above type, a liquid carrier such as a fatty glyceride or mixtures of fatty glycerides. Dosage forms may also include oral suspensions.

Various other materials may be present as coatings or to modify the physical form of a dosage unit. For instance, tablets may be coated with shellac, sugar or both. A syrup or elixer may contain, in addition to the active ingredient(s), sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and a flavoring such as cherry or orange flavor.

The pharmaceutical forms suitable for injectable use include sterile solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sufficiently fluid to enable incorporation into a syringe and injection therefrom and must be substantially stable under the conditions of manufacture and storage. In addition, the form must be substantially sterile and must be preserved against contamination of microorganisms such as bacteria and fungi. Sterilization may be achieved by filtration through microorganism retaining filters, by incorporating sterilizing agents into the compositions, or by irradiating or heating the compositions wherein such irradiation or heating is both appropriate and compatible with the applicable formulation.

Additional pharmaceutical forms may include suppositories, sublingual tablets, topical dosage forms and the like, and these may be prepared according to methods which are commonly known by those skilled in the art.

The present invention provides use of an agonist of the LPL receptors and a ligand of a class III tyrosine kinase receptor for modulating spontaneous differentiation of a stem cell having a lysophospholipid (LPL) receptor and PDGF receptors.

The present invention also provides use of a ligand of a class III tyrosine kinase receptor in modulating spontaneous differentiation of a stem cell.

The present invention further provides use of an agonist of the LPL receptor for modulating spontaneous differentiation of a stem cell having a lysophospholipid (LPL) receptor.

Preferably, the LPL receptor is selected from the group consisting of  
5 S1P1, S1P2 and S1P3.

Typically the agonist is a phospholipid.

The phospholipid or lysophospholipid may have a sphingosine backbone, and particularly, the lysophospholipid may be a phosphorylated amino alcohol. Preferably the agonist is selected from the group consisting  
10 of S1P, dihydro S1P, LPA, PAF and SPC or functional equivalents thereof.

In a highly preferred form of the invention the lysophospholipid is sphingosine-1-phosphate (S1P) or a functional equivalent thereof.

Typically the ligand is a PDGF or functional equivalent thereof.

The tyrosine kinase receptor may be PDGFR- $\alpha$  or PDGFR- $\beta$ .

15 In a preferred embodiment the PDGF is PDGF $\alpha\alpha$ , PDGF $\alpha\beta$  or PDGF $\beta\beta$  which bind to the two types of receptors.

TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters may also be used as compounds that have additive or synergistic effects with S1P in other cell types.

20 The stem cell may be derived from foetal tissue or adult tissue.

The stem cell is typically an ES cell. Preferably the stem cell is a hES cell.

The present invention provides use of an agonist of the LPL receptor and a ligand of a class III tyrosine kinase receptor in producing a population  
25 of proliferating undifferentiated stem cells from a stem cell.

The present invention also provides use of a ligand of a class III tyrosine kinase receptor in producing a population of proliferating undifferentiated stem cells from a stem cell.

The present invention further provides use of a method of an agonist  
30 of the LPL receptor in producing a population of proliferating undifferentiated stem cells from a stem cell.

Preferably, the LPL receptor is selected from the group consisting of S1P1, S1P2 and S1P3.

Typically the agonist is a phospholipid.

Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF and SPC or functional equivalents thereof.

In a highly preferred form of the invention the lysophospholipid is  
 5 sphingosine-1-phosphate (S1P) or a functional equivalent thereof.

Typically the ligand is a PDGF or functional equivalent thereof.

The tyrosine kinase receptor may be PDGFR- $\alpha$  or PDGFR- $\beta$ .

In a preferred embodiment the PDGF is PDGF $\alpha\alpha$ , PDGF $\alpha\beta$  or PDGF $\beta\beta$  which bind to the two types of receptors.

10 The ligand may also be TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters.

The stem cell may be derived from foetal tissue or adult tissue.

The stem cell is typically an ES cell. Preferably the stem cell is a hES cell.

15 Another aspect of the present invention is use of a composition containing an agonist of a LPL receptor and a ligand of a class III tyrosine kinase receptor in a method of treating or preventing a disorder of stem cell differentiation.

The present invention also provides use of a composition containing a  
 20 ligand of a class III tyrosine kinase receptor in a method of treating or preventing a disorder of stem cell differentiation.

The agonist is typically a phospholipid. The phospholipid may be a lysophospholipid and may have a sphingosine backbone. Preferably the agonist is selected from the group consisting of S1P, dihydro S1P, LPA, PAF  
 25 and SPC. S1P and dihydro S1P are lysophospholipids with a sphingosine backbone, as is SPC, while LPA is a lysophospholipid with a glycerol backbone, and PAF is a phospholipid with a glycerol backbone.

The tyrosine kinase receptor may be PDGFR- $\alpha$  or PDGFR- $\beta$  and the ligand a PDGF or functional equivalent thereof.

30 In a preferred embodiment the PDGF is PDGF $\alpha\alpha$ , PDGF $\alpha\beta$  or PDGF $\beta\beta$ .

The method may also include use of TNF  $\alpha$ , NGF (nerve growth factor), muscarinic acetylcholine agonists, serum or phorbol esters - which

again are compounds that have additive or synergistic effects with S1P in other cell types.

### Abbreviations

5            dH-S1P: dihydro-sphingosine-1-phosphate; EDG: endothelial differentiation gene; ERK: extracellular signal-regulated kinase; MAP kinase: mitogen-activated protein kinase; MEF: mouse embryonic fibroblasts; hES cells: human embryonic stem cells; LPA: lysophosphatidic acid; LPL: lysophospholipid; PAF: platelet-activated factor; PCNA: proliferating cell  
10 nuclear antigen; PDGF: platelet-derived growth factor; PDGFR: platelet-derived growth factor receptor; S1P: sphingosine-1-phosphate; SPC: sphingosylphosphorylcholine; SPK: sphingosine kinase.

### Brief Description of the Accompanying Figures

15            FIGURE 1: shows hES cells are target of S1P, LPA and PDGF. RT-PCR for LPL receptors (**A, B**), PDGFR- $\alpha$  (alpha) and PDGFR- $\beta$  (beta) (**C**), SPK-1 and SPK-2 (**D**), with (+) or without (-) RT. Immunostaining of hES cells with Hoechst 33342 (**E, H**), PDGFR- $\alpha$  (**F**) or PDGFR- $\beta$  (**I**) and GCTM-2 (**G, J**) antibodies. S1P, LPA and PDGF stimulate ERKs phosphorylation in hES  
20 cells. (K) Western blots experiment were performed using protein lysate from hES cells. Cells were pre-treated or not with U0126 (30  $\mu$ M, 1 hr) and incubated for 5 min in the absence (C, control) or presence of S1P (S, 10  $\mu$ M), LPA (L, 50 M) or PDGF (P, 20 ng/ml). The phosphorylation of Erk1 and Erk2 (P-Erk1 and P-Erk2) was assessed by immunoblotting with a polyclonal  
25 anti-active MAP kinase as described in Materials and Methods. After a stripping procedure, the same blots were reprobed with a monoclonal anti-MAP kinase, allowed the detection of Erk1 and Erk2. These data are representative of results from at least 3 independent experiments.

30            FIGURE 2 shows S1P and PDGF inhibit the spontaneous differentiation of hES cells. (A) hES cells grown with MEF, before the depletion of serum

from the medium. (B, C, D, E) hES cells grown without serum after 8 days, in the absence (B) or in the presence of S1P (10  $\mu$ M) (C), PDGF (20 ng/ml) (D), S1P (10  $\mu$ M) plus PDGF (20 ng/ml) (E). (F) hES cells grown without serum, in the presence or in the absence (control) of S1P (10  $\mu$ M), PDGF (20 ng/ml),

5 S1P (10  $\mu$ M) plus PDGF (20 ng/ml). In A-E, data are representative of at least 3 independent experiments. In F, data expressed as percentages of alkaline phosphatase activity in absence of serum for eight days (% of control), are the means  $\pm$  SEM of at least 2 independent experiments, each run in triplicate.

10 FIGURE 3 shows S1P and PDGF inhibit the spontaneous differentiation of hES cells independently of MEF. hES cells mechanically dissociated and cultivated for 4 days in the absence (C, control) or presence of S1P (10  $\mu$ M) or/and PDGF (20 ng/ml) in a media depleted in serum. (A) Quantification of the number of GCTM2+ cells. (B) Quantification of the number of

15 PCNA+/GCTM2+ cells. These data are the mean  $\pm$  SEM of results obtained in at least 3 independent experiments.

FIGURE 4 shows hES cells are target of S1P, LPA and PDGF. RT-PCR for LPL receptors (A, B), PDGFR- $\alpha$  (alpha) and PDGFR- $\beta$  (beta) (C), SPK-1 and SPK-2 (D), with (+) or without (-) RT. Immunostaining of hES cells with

20 Hoechst 33342 (E, H), PDGFR- $\alpha$  (F) or PDGFR- $\beta$  (I) and GCTM-2 (G, J) antibodies. S1P, LPA and PDGF stimulate ERKs phosphorylation in hES cells.

FIGURE 5 shows S1P and PDGF inhibit the spontaneous differentiation of hES cells in the absence of serum. (A-C) hES cells with or without (control)

25 the indicated agonists. Dihydro-S1P: DHS1P. (D) Sphingosine kinase activity measurement following incubation of hES cells with PDGF.

FIGURE 6 shows characterization of hES cells. (A) hES cells grown in the presence of S1P + PDGF, passage 14. (B) RT-PCR using mRNA from hES cells grown in the presence of S1P and PDGF using specific primers for

30 Oct-4, cripto, SPK1 and SPK2, with (+) or without (-) RT, passage 7.

Immunostaining of hES cells grown in the presence of S1P + PDGF with GCTM-2 (C), Oct-4 (D), TG-30 (E) or TRA-1-60 (F), passage 13. (G) Karyotyping of hES cells grown in the presence of S1P + PDGF, passage 8. (H) Neuronal differentiation into neurospheres. (I)  $\beta$ tubulin immunostaining

5       FIGURE 7 shows Edg receptor mRNAs are expressed in hES cells. RT-PCR experiments were performed using mRNA isolated from hES cells using specific primers for human Edg receptors. In each case, experiments were conducted either in the presence (+) or absence (-) of reverse transcriptase. The RT-PCR products were separated by electrophoresis on  
10   1.5% agarose gel and revealed by ethidium bromide fluorescence. Molecular sizes (in bp) of the products were calculated using 1 kB plus DNA ladder markers (M). These data are representative of at least 6 independent experiments, each carried out on mRNAs prepared from different cultures of hES cells.

15       FIGURE 8 shows S1P inhibits the spontaneous differentiation of hES cells. (A) hES cells grown with feeder, before the depletion in serum. (B) hES cells grown without serum after 8 days (B, C) and 12 days (D, E), in absence (B, D) or presence of S1P (C, E, 10  $\mu$ M). These data are representative of at least 3 independent experiments.

20       FIGURE 9 shows S1P inhibits the spontaneous differentiation of hES cells. Double staining experiments were performed using antibodies for PCNA and GCTM-2. These data are representative of at least 3 independent experiments.

FIGURE 10 shows S1P stimulates ERKs phosphorylation in hES cells.  
25   Western blots experiments were performed using protein lysate from hES cells. (A) Cells were pre-treated or not with U0126 (30  $\mu$ M, 1 hr) and incubated for 5 min in the absence (C, control) or presence of S1P (10  $\mu$ M). (B) Cells were incubated for different time periods in the absence or presence of S1P (10  $\mu$ M). (C) Cells were incubated for 5 min with various  
30   concentrations of S1P. The phosphorylation of Erk1 and Erk2 (P-Erk1 and P-Erk2) was assessed by immunoblotting with a polyclonal anti-active MAP kinase as described in Materials and Methods. After a stripping procedure,

the same blots reprobed with a monoclonal anti-MAP kinase, allowed the detection of Erk1 and Erk2. These data are representative of at least 3 independent experiments.

The invention will now be more fully described with reference to the  
5 following non-limiting Examples.

## **Best Method and Other Methods of Carrying out the Present Invention**

### **EXAMPLE 1**

10

#### ***Cell culture***

hES-3 cells were cultured as previously described <sup>1</sup>. The serum-free culture medium consisted of DMEM (without sodium pyruvate, glucose 4500 mg/l, Invitrogen, Mount Waverley, VIC, Australia) supplemented with  
15 insulin/transferrin/selenium 1%,  $\beta$ -mercaptoethanol 0.1 mM, NEAA 1 %, glutamine 2 mM, Hepes 25 mM, penicillin 50 U/ml and streptomycin 50 mg/ml (all from Invitrogen). Medium was changed every 2 days and cells were passaged every week. S1P and dihydro-S1P were obtained from Biomol (Plymouth Meeting, PA, USA) and were dissolved in methanol. LPA  
20 was obtained from Sigma (Castle Hill, NSW, Australia) and was dissolved in ethanol. Extemporaneous dilutions of all lipids were made in water containing 0.1 % fatty acid-free bovine serum albumin (BSA) (Sigma). Human PDGF-AB, PDGF-AA, PDGF-BB were from PreproTech Inc. (Rocky Hill, NJ, USA).

#### ***RT-PCR experiments.***

25

Total RNA was extracted from hES cells and reverse transcribed (RT) as previously described <sup>1</sup>. The cDNA samples were amplified by PCR with sense and antisense primers (Sigma) designed for the specific detection of mouse (data not shown) or human DNA target sequences (Table 1) using

Taq DNA polymerase (Biotech International Ltd, Perth, WA, Australia) as previously described <sup>18</sup>. The specific amplified DNA fragments were sized by electrophoresis on 1.5 % (w/v) agarose gel and stained with ethidium. Molecular sizes (bp) were calculated using 1 kb plus DNA ladder markers (M). The amplicons were purified and sequenced. Experiments were performed on hES-2 and hES-3.

**Table 1 : Sense and antisense primers**

Gene	sense and antisense primers	Size (bp)	Annealing temp (°C)	References
S1P <sub>1</sub>	CCACAACGGGAGCAATAACT GTAAATGATGGGGTTGGTGC	480	52	<sup>2</sup>
S1P <sub>2</sub>	CCAATACCTTGCTCTCTCTGGC CAGAAGGAGGATGCTGAAGG	502	52	<sup>2</sup>
S1P <sub>3</sub>	TCAGGGAGGGCAGTATGTTC CTGAGCCTTGAAGAGGATGG	505	52	<sup>2</sup>
S1P <sub>4</sub>	CGGCTCATTGTTCTGCACTA GATCATCAGCACCGTCTTCA	701	52	<sup>2</sup>
S1P <sub>5</sub>	TTCTGATACCAGAGTCCGGG CAAGGCCTACGTGCTCTTCT	460	52	<sup>2</sup>
LPA <sub>1</sub>	GCTCCACACACGGATGAGCAACC GTGGTCATTGCTGTGAACTCCAGC	621	56	<sup>3</sup>
LPA <sub>2</sub>	AGCTGCACAGCCGCCTGCCCCGT TGCTGTGCCATGCCAGACCTTGTC	775	56	<sup>3</sup>



LPA <sub>3</sub>	CCATAGCAACCTGACCAAAAAGAG TCCTTGTTAGGAGTAGATGATGGGG	482	56	3
PDGFR $\alpha$	ATCAATCAGCCCAGATGGAC TTCACGGGCAGAAAGGTACT	891	58	4
PDGFR $\beta$	AATGTCTCCAGCACCTTCGT AGCGGATGTGGTAAGGCATA	698	58	4
Crypto	CAGAACCTGCTGCCTGAATG GTAGAAATGCCTGAGGAAACG	185	55	
SPK-1	ACCCATGAACCTGCTGTCTC CAGGTGTCTTGAACCCACT	227	55	
SPK-2	TGGCAGTGGTGTAGAACC CAGTCAGGGCGATCTAGGA	200	55	
Oct-4	CGTTCTCTTTGGAAAGGTGTTT ACACTCGGACCACGTCTTTC	320	55	5

### ***Immunofluorescence.***

- 5            In some experiments, hES-3 cells plated onto 8-well chamber slides, with or without MEF, were fixed in ethanol or paraformaldehyde (for PDGFR) the day after plating. In others, hES-3 cells were mechanically dissociated, in order to obtain a monolayer culture and then plated onto 8-well chamber slides without MEF and were fixed in ethanol 4 days after the first treatment.
- 10 Immunostaining was performed using the following antibodies: anti human PDGFR- $\alpha$  or PDGFR- $\beta$  (R&D Systems Inc.), GCTM-2, and/or PCNA (Chemicon, Boronia, VIC, Australia), TRA-1-60, Oct-4. Nuclei were evidenced by Hoechst-33342. Slides were mounted and observed by

fluorescent microscopy with a Leica microscope at X10, X20 and X40. Specificity was verified by the absence of any staining in the negative controls. In some experiments, cells were counted to determine the ratio of GCTM-2 positive (GCTM2+), PCNA positive (PCNA+) and GCTM2+/PCNA+ cells within the global population.

#### ***GCTM-2 quantification.***

hES-3 cells plated with MEF, were fixed in ethanol and immunostained with GCTM-2 and then with an alkaline phosphatase-coupled secondary antibody (Dako). The activity of alkaline phosphatase was detected by adding a solution of 4-nitrophenyl phosphate (Roche, Mannheim, Germany), followed by reading the optical density (OD) at 405 nm. In order to validate the technique as a relevant indicator of the proportion of GCTM-2 positive cells, standard curves were done with the teratocarcinoma cell line GCT27C4, known to express GCTM-2. This showed a linear correlation between the number of cells and the OD read at 405 nm (data not shown).

#### ***Western blot analysis.***

hES-3 cells plated without MEF for 24 hrs were depleted of serum for a further 18 hrs. Cells pre-treated or not with U0126 (Sigma, 30  $\mu$ M, 1 hr), were incubated in the presence of the different agents for 5 min and were lysed by removal of the supernatants and addition of a reducing loading buffer containing 1 mM sodium orthovanadate (Sigma). Protein lysates (approx. 80  $\mu$ g) were separated by SDS-polyacrylamide gel electrophoresis (10 % polyacrylamide, w/v), transferred to nitrocellulose (Hybond-nitrocellulose, Amersham) and immunoblotting was carried out using rabbit polyclonal anti-active mitogen-activated protein (MAPK) antibodies raised against a dually phosphorylated MAPK peptide (Promega, Madison, WI, USA). Peroxidase-coupled secondary antibody (Dako) was detected by exposure of autoradiographic films in the presence of a chemiluminescent detection reagent (ECL, Amersham). Stripping of antibodies was achieved and membranes were then reprobed with rabbit polyclonal anti-ERK1/2 antibodies (Promega), and then with peroxidase-coupled secondary

antibodies (Dako). Membranes probed with either rabbit polyclonal anti-active p38 (Promega) or rabbit polyclonal anti-active JNK (Promega) antibodies were also performed, using the same procedure as described above.

5

#### ***Protein quantification.***

hES-3 cells were lysed and the amount of proteins was determined using a colorimetric assay based on the Bradford dye-binding test (Bio-Rad Laboratories, Regents Park, NSW, Australia).

10

#### ***Statistical analysis.***

Each set of experiments was performed at least 3 times ( $n$  refers to number of independent experiments performed on different cell cultures). Data are expressed as the mean  $\pm$  SEM. Significance of the differences was evaluated by using the ANOVA followed by Student-Newman Keuls test. Values of  $P < 0.05$  were considered significant and were respectively indicated by \*.

15

## **RESULTS**

hES cells (Figure 1A) expressed mRNA transcripts for three S1P receptors: S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> and for each of LPA receptors: LPA<sub>1</sub>, LPA<sub>2</sub> and LPA<sub>3</sub> (Figure 1B), while these cells did not express mRNA for S1P<sub>4</sub> and S1P<sub>5</sub> (data not shown). hES cells also expressed mRNA transcripts for PDGFR- $\alpha$  (Figure 1C) and PDGFR- $\beta$  (Figure 1C) as well as the corresponding proteins, as revealed by immunostaining (Figure 1E-J). MEF expressed S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>, LPA<sub>1</sub> and LPA<sub>2</sub>, PDGFR- $\alpha$  and PDGFR- $\beta$  but neither S1P<sub>4</sub>, S1P<sub>5</sub> nor LPA<sub>3</sub> (data not shown), as previously shown by others<sup>6-8</sup>. Because the MAP kinases ERKs are implicated in cell proliferation and differentiation, we examined the effects of S1P, LPA and PDGF-AB (PDGF) on their activation in hES cells. After 5 min, S1P, LPA and PDGF stimulated the phosphorylation of ERKs in hES cells (Figure 1K), an effect that was totally inhibited in presence of the MEK inhibitor U0126 (30  $\mu$ M) (Figure 1K).

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Next, it was examined whether S1P, LPA and PDGF could modulate the fate of hES cells. When hES cells were grown on MEF, in a serum-free culture media, they spontaneously differentiated. As shown in Figure 2, after 8 days in such conditions (control), the colonies were bigger than those observed before the removal of serum (Figure 2A) and hES cells gave rise to different kinds of cells (Figure 2B). After 8 days, LPA (up to 50  $\mu$ M) did not have an obvious effect on growth of the colonies, as ascertained by morphological (data not shown) whilst in the presence of either S1P (10  $\mu$ M) or PDGF (20 ng/ml), the colonies appeared flatter and less differentiated as compared to the control condition (Figure 2C, 2D). Thus, after 8 days of treatment, when GCTM-2 levels of cells were quantified by measuring the activity of alkaline phosphatase, cells treated with S1P or PDGF were respectively  $16.6 \pm 4.1$  % (n=7) and  $16.6 \pm 7.0$  % (n=7) more GCTM2+ than the control cells (Figure 2F). Strikingly, the co-incubation of both S1P (10  $\mu$ M) and PDGF (20 ng/ml) induced a strong inhibition of spontaneous differentiation, not observed in the presence of one or the other agent (Figure 2E) with a higher percentage of GCTM2+ cells of  $40.1 \pm 7.5$  % (n=7) than in the control cells (Figure 2F). As GCTM-2 is a stem cell marker, these results suggest that the combination of PDGF and S1P in a serum-free culture media strongly prevents the spontaneous differentiation of hES cells.

In order to identify the effects of S1P and PDGF on hES cells, we carried out experiments in which we forced the cells to differentiate, by 1) mechanically dissociating them before plating and 2) growing them in the absence of MEF and serum. S1P or/and PDGF were added to the culture medium and their effects on differentiation and proliferation were quantified by immunostaining the cells with PCNA, a marker of proliferation, and GCTM-2 (Figure 3). After 4 days in medium without serum, most of the

control cells were differentiated, with only  $30.8 \pm 7.7$  % (n=13) of GCTM2+ cells (Figure 3A). By contrast, when either S1P (10  $\mu$ M) or PDGF (10 ng/ml) was added to the medium,  $47.9 \pm 3.8$  % (n=13) or  $53.7 \pm 13.2$  % (n=3) of the cells respectively were GCTM2+, and  $53.7 \pm 3.5$  % (n=3) of the cells were GCTM2+ in presence of both S1P and PDGF. Within the hES cell population, a large proportion expressed PCNA, showing that the majority of these stem cells still proliferated (Figure 3B). However, there was no statistically significant difference in the proliferating rate of hES cells between the control cells and the ones treated with either S1P or/and PDGF (Figure 3B). Altogether, these data suggest that S1P and PDGF mostly act on the differentiation of hES cells grown in the absence of serum rather than on the proliferating state of hES cells. Moreover, because the hES cells were cultivated in absence of MEF, these experiments clearly show that S1P and PDGF are able to directly target the hES cells.

We next investigated the effect of dihydrosphingosine-1-phosphate (dihydro-S1P, 10  $\mu$ M), an S1P analogue that can only mimics the receptor-dependent effects of S1P. By measuring the GCTM2 levels of the cells, we showed that the effect seen in presence of S1P and PDGF was mimicked by dihydro-S1P and PDGF ( $125.7 \pm 9.7$  % of control (n=3)), demonstrating that S1P's effect is receptor-dependent (Figure 2F). We then investigated which isoform of PDGF was the most potent in inhibiting the spontaneous differentiation of hES cells. When added with S1P, the isoform BB was the most potent ( $182.0 \pm 26.0$  % of control (n=2)), followed by AB ( $125.7 \pm 9.7$  % of control (n=3)), while AA elicited little effect ( $120.5 \pm 4.5$  % of control (n=2)) (Figure 2F).

### ***Passaging***

The hES cells have successfully been passaged through at least 18 passages in PDGF and S1P, with no serum. After passage 13 the cells have stained positive for the stem cell markers GCTM-2, Oct-4 and TG30. After passage 7 the cells expressed mRNA for SPK1 and SPK2 showing the  
 5 probable expression of the enzymes as well as the stem cell markers Oct-4, and Crypto. After passage 8: karyotyping of hES cells – is being carried out to show that these cells when cultured in serum free conditions with PDGF and S1P have maintained a normal karyotype.

## DISCUSSION

10 Since hES cells spontaneously differentiate in culture, a phenomenon that leads to a loss of their pluripotency, the identification of the compounds that are able to prevent this differentiation is of particular interest. In this study, we describe for the first time that hES cells are targets of S1P, LPA and PDGF.

15 As revealed by RT-PCR analysis, these cells express the mRNA for the receptors S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>, LPA<sub>1</sub>, LPA<sub>2</sub> and LPA<sub>3</sub>. Referring to studies performed in rodent or in human, these receptors are widely expressed in the body (for reviews see <sup>9,10</sup>). The absence of expression of S1P<sub>4</sub> and S1P<sub>5</sub> in these cells is in accordance with the fact that these receptors seem to be  
 20 mostly expressed in highly differentiated tissues, such as lymphoid tissue for S1P<sub>4</sub> <sup>11</sup> and in brain's white matter for S1P<sub>5</sub> <sup>12</sup>. Moreover, hES cells express the PDGF-receptors  $\alpha$  and  $\beta$ , as revealed by RT-PCR and immunostaining. In hES cells, the addition of both PDGF and S1P inhibit very strongly the spontaneous differentiation, suggesting that these two molecules do cross  
 25 talk. These combined effects could be attributed to the fact that 1) PDGF stimulates the formation of intracellular S1P which would then act as a second messenger, for instance in the regulation of calcium homeostasis <sup>13</sup> and in the suppression of apoptosis, as shown in fibroblasts <sup>14</sup> and other cell

types <sup>15,16</sup>, but up to now the intracellular targets of S1P remain unclear; 2) S1P acts extracellularly through its receptors, and thus activates different intracellular signalling pathways, such as the MAP kinases, involved in cell proliferation. The presence of both intracellular and extracellular S1P might  
 5 then lead to a stronger inhibition of differentiation than the ones observed in presence of either S1P or PDGF. Also reported is a new cross link between PDGF and S1P signals, in which both molecules need to be present. Such a mechanism has recently been described for the first time by Katsuma *et al.* (2002) <sup>17</sup> in mesangial cells.

10 As shown by others, S1P, LPA and PDGF receptors are expressed in MEF <sup>7</sup> and these molecules are able to regulate multiple signalling pathways. Thus, Ishii *et al.* (2001) demonstrated that in these cells, S1P activates phospholipase C, inhibits the production of cAMP and activates Rho <sup>7</sup>. In MEF, PDGF stimulates migration. The effect observed in presence of PDGF  
 15 and S1P on hES cells might be in part due to an effect through the MEF.

S1P, LPA and PDGF are all present in serum from different species, including bovine and human. However, the concentration of these molecules varies from one species to another. Thus, it is believed that this could explain the commonly observed phenomenon with current cell culturing techniques  
 20 where there is not only species dependant variation in the performance of serum used to supplement cell culture systems but also intra-species batch to batch variations as well.

Altogether, these data suggest that within the lipids and the proteins present into the serum, both S1P and PDGF are key elements in the  
 25 regulation of spontaneous differentiation of hES cells. Identification of compounds having an ability to inhibit differentiation allows the design of

simple culture media more suitable for hES cell propagation. Moreover, in a therapeutic view, it is important to determine compounds that allow cultivation of hES cells in a serum-free environment.

## EXAMPLE 2

### 5 ***Cell culture.***

hES-3 cells were cultured as previously described <sup>1</sup>. The serum-free culture medium consisted of DMEM (without sodium pyruvate, glucose 4500 mg/l, Invitrogen, Mount Waverley, VIC, Australia) supplemented with insulin/transferrin/selenium 1%,  $\beta$ -mercaptoethanol 0.1 mM, NEAA 1 %, 10 glutamine 2 mM, Hepes 25 mM, penicillin 50 U/ml and streptomycin 50 mg/ml (all from Invitrogen). Media was changed every 2 days and cells were passaged every week. S1P and dihydro-S1P were obtained from Biomol (Plymouth Meeting, PA, USA). LPA was obtained from Sigma (Castle Hill, NSW, Australia). Extemporaneous dilutions of all lipids were made in water 15 containing 0.1 % fatty acid-free bovine serum albumin (BSA) (Sigma). S1P and dihydro-S1P were used at 10 mM. Human PDGF-AB, PDGF-AA, PDGF-BB were from PreproTech Inc. (Rocky Hill, NJ, USA) and were used at 20 ng/ml.

### ***RT-PCR experiments.***

20 Total RNA was extracted from hES cells and reverse transcribed (RT) as previously described <sup>1</sup>. The cDNA samples were amplified by PCR with sense and antisense primers (Sigma) designed for the specific detection of mouse (data not shown) or human DNA target sequences (Table 1) using Taq DNA polymerase (Biotech International Ltd, Perth, WA, Australia) as 25 previously described <sup>18</sup>. The specific amplified DNA fragments were sized by



electrophoresis on 1.5 % (w/v) agarose gel and stained with ethidium. Molecular sizes (bp) were calculated using 1 kb plus DNA ladder markers (M). The amplicons were purified and sequenced. Experiments were performed on hES-2 and hES-3.

## 5 ***Immunofluorescence.***

Cells were fixed in paraformaldehyde 4% (for PDGFR staining) or 100 % ethanol and immunostained as previously described <sup>1</sup> using the following antibodies: anti-human PDGFR- $\alpha$  or PDGFR- $\beta$  (R&D Systems Inc., Minneapolis, MN, USA), GCTM-2 (this laboratory), TRA-1-60 (gift from P. Andrews, University of Sheffield), Oct-4 (Santa Cruz, CA, USA), TG-30 (this laboratory). Nuclei were counter-stained with Hoechst-33342 (Chemicon). Specificity was verified by the absence of any staining in the negative controls.

## ***Sphingosine kinase activity.***

hES-3 cells plated without MEF for 24 hr and depleted of serum for a further 18 hr were incubated in the presence of PDGF (20 ng/ml) for various time periods and were harvested and lysed by sonication (2 W for 30 s at 4°C) in lysis buffer containing 50 mM Tris/HCl (pH 7.4), 10% glycerol, 0.05% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM EDTA and protease inhibitors (Complete™, Roche, Mannheim, Germany). SPK activity was determined using D-erythro-sphingosine and [ $\alpha$ <sup>32</sup>P]ATP as substrates, as previously described <sup>19</sup>. Protein concentrations in cell homogenates were determined with Coomassie Brilliant Blue reagent (Bio-Rad, Regent Park, NSW, Australia) using bovine serum albumin as standard.

## ***GCTM-2 quantification.***

Cells were fixed in 100 % ethanol and immunostained with GCTM-2 followed by alkaline phosphatase-coupled secondary antibodies (Dako). Alkaline phosphatase activity was detected by adding a solution of 4-nitrophenyl phosphate (Roche), and the concentration of the reaction product was determined by reading the optical density (OD) at 405 nm. In order to validate the technique as an accurate indicator of the proportion of GCTM-2 positive cells, standard curves were carried out with the embryonal carcinoma cell line GCT27C4, known to express GCTM-2<sup>20</sup>. This showed a linear correlation between the number of cells and the OD read at 405 nm (data not shown).

#### ***Neuronal induction of hES cells.***

hES-3 cells (passages 11, 13-15) were differentiated into noggin cells by a noggin treatment then into neurospheres and last into neurons as previously described in PCT/AU01/00735.

#### ***Statistical analysis.***

All experiments were performed at least 3 times. Data are expressed as the mean  $\pm$  SEM of at least 3 independent experiments. Significance of the differences was evaluated using an ANOVA followed by Student-Newman Keuls test. Values of  $P < 0.05$  were considered significant (\*).

#### ***Results***

hES cells expressed mRNA transcripts for three S1P receptors: S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> and for each of the LPA receptors (Fig. 4A-B). However these cells did not express mRNA for S1P<sub>4</sub> and S1P<sub>5</sub>. Contrary to mouse embryonic stem cells, hES cells expressed mRNA transcripts for PDGFR- $\alpha$  and PDGFR- $\beta$  (Fig. 4C) as well as the corresponding proteins, as revealed by immunostaining (Fig. 4E-J). As previously shown by others<sup>6-8,18,19</sup>, we

show that MEF expressed S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub>, LPA<sub>1</sub>, LPA<sub>2</sub>, PDGFR- $\alpha$  and PDGFR- $\beta$ . Thus in a co-culture system, S1P, LPA and PDGF could be active on either cell type.

We next examined whether S1P, LPA and PDGF could affect growth or differentiation of hES cells. When hES cells were grown on MEF in a serum-free culture medium, they spontaneously differentiated into different kinds of cells. After 2 weeks in a serum-free media, LPA (up to 50  $\mu$ M) had no obvious effect on size or morphology of hES cell colonies whilst in the presence of either S1P (10  $\mu$ M) or PDGF-AB (PDGF, 20 ng/ml), the colonies appeared flatter and less differentiated as compared to the controls. Moreover, the co-incubation of S1P and PDGF induced a strong inhibition of spontaneous differentiation. To quantify these effects, we used an ELISA-based assay to measure expression of the stem cell surface antigen GCMT-2 (GCTM2+) in cells treated for 2 weeks with different agonists. Thus, cells treated with S1P or PDGF were respectively  $18.0 \pm 17.0$  % (n=3) and  $50.3 \pm 18.4$  % (n=3) more GCTM2+ than the controls and the ones treated with both S1P and PDGF were  $152.7 \pm 54.9$  % (n=3) more GCTM2+ than the controls (Fig. 5A). Using the same technique, we showed that cells treated with S1P and either PDGF-AA or PDGF-BB showed a GCTM2 expression similar to the one observed with S1P and PDGF (PDGF-BB:  $294.3 \pm 77.3$  % of control, n=3, PDGF-AA:  $220.3 \pm 49.0$  % of control, n=3; Fig. 5A). Moreover, the effect of S1P in combination with PDGF was mimicked by the use of dihydrosphingosine-1-phosphate (dihydro-S1P, 10 mM), a S1P analogue that mimics its receptor-dependent effects, in combination with PDGF ( $227.0 \pm 59.9$  % of control (n=3), Fig. 5A). Furthermore, dihydro-S1P on its own had a more potent effect on hES cells than S1P ( $223.0 \pm 27.0$  % of control, n=3; Fig. 5A). Together, these results suggest that the combination of PDGF and

S1P in a serum-free culture medium prevents the spontaneous differentiation of hES cells. This effect is dependent upon S1P's receptors and both PDGFRs, as PDGF-AA only binds to PDGFR- $\alpha$  while PDGF-AB and PDGF-BB bind to both receptors. Moreover, treatment of hES cells with the MAP kinase kinase inhibitor U0126 (Promega, 10  $\mu$ M) for 7 days, totally inhibited the effect of PDGF and S1P on GCTM2 expression (Fig. 5B), strongly suggesting that the activation of the extracellular signal-regulated kinases is required to maintain hES cells undifferentiated. As SPK is a key molecule in PDGF signalling pathways, we verified the presence of SPK transcripts in hES cells and showed expression of both SPK-1 and SPK-2 mRNA (Fig. 4D). We next investigated if PDGF modulates SPK activity in hES cells (Fig. 5D). PDGF (20 ng/ml) enhanced in a time-dependent manner the SPK activity in hES cells (Fig. 5D). This effect lasted for at least 60 min and SPK activity reached 1.6 fold the basal values ( $75.3 \pm 3.92$  nmol/min/mg,  $n=3$ ) after 30 min of incubation (Fig. 5D). In contrast, PDGF (20 ng/ml) did not induce a significant statistical activation of SPK in MEF. Moreover, treatment of hES cells with dimethylsphingosine (DMS, 3  $\mu$ M, Fig. 5C), a non-specific inhibitor of SPK, for 7 days, inhibited the effect of PDGF and S1P, suggesting an involvement of SPK in the maintenance of hES in an undifferentiated state.

To date, hES cells have been grown in a serum-free medium supplemented with S1P (10  $\mu$ M) and PDGF (20 ng/ml) for 19 passages. As these cells still express SPK-1 and SPK-2 mRNA (Fig. 6B), we can expect the PDGF-activation of SPK to be involved in the propagation of hES cells. RT-PCR studies showed that hES cells expressed the mRNA for Oct-4 and cripto (Fig. 6B), and immunostaining showed immunoreactivity to the stem cell markers GCTM-2, Oct-4, TG-30 and Tra-1-60 (Fig. 6C-F). These hES cells retained a normal karyotype (Fig. 6G). Moreover, these HES cells still

responded to noggin treatment and were able to form neurospheres (Fig. 6H) and neuronal cells as ascertained by immunostaining for  $\beta$ tubulin (Fig. 6I), Map2, nestin, synaptophysin, N-cam and NF200 (Pera et al submitted). Altogether, these data demonstrate that HES cells grown in the presence of S1P and PDGF retain the characteristics of HES cells propagated in normal serum conditions.

### **Discussion**

In this study, we show for the first time that hES cells are targets of S1P, LPA and PDGF and we also show an interaction between S1P and PDGF signal, in that extracellular S1P and PDGF need to be present together to exert a potent biological effect. Katsuma *et al.* (2002)<sup>17</sup> reported a similar mechanism in mesangial cells where application of S1P and PDGF increases proliferation. In hES cells the addition of both S1P and PDGF maintains these cells in the undifferentiated state, and still allows them to follow differentiation. These combined effects could be attributed to the fact that (i) S1P acts extracellularly through its receptors to modulate intracellular signalling pathways; (ii) and that PDGF stimulates the formation of intracellular S1P which would either be secreted or act as an intracellular messenger, for instance in the regulation of calcium homeostasis<sup>13</sup> and in the suppression of apoptosis, as shown in fibroblasts<sup>14</sup> and other cell types<sup>15,16</sup>. Whether S1P is secreted or acts as a second messenger needs to be further investigated. However, because the maintenance of hES cells in an undifferentiated state only occurs in the presence of both PDGF and S1P, we could expect that intracellular S1P, produced in response to PDGF, acts within the cells, as its cell-surface receptors are likely to have already been engaged by S1P previously added to the culture media. To our knowledge, this study is the first one to report a cross-talk involving S1P and two isoforms of PDGFR, instead of only PDGFR- $\beta$ . These data demonstrate that

S1P and PDGF are key elements in the regulation of spontaneous differentiation of hES cells. Their identification as compounds having an ability to inhibit differentiation allows the design of a simple serum-free culture medium more suitable for hES cell propagation.

- 5           The following materials and methods relate to Examples 3 to 5.

### **Reagents**

S1P AND LPA were obtained from Biomol (Plymouth Meeting, PA, USA) and were dissolved in methanol. Freshly prepared dilutions of agonists were made in water containing 0.1% fatty acid-free bovine serum albumin  
10 (BSA) (Sigma). Protease, sodium orthovanadate and U0126 were from Sigma. was from Calbiochem (San Diego, CA, USA). *Pertussis* Toxin (PTX) was from List Biological Laboratories (Campbell, CA, USA). GCTM-2, Oct-4, PCNA, Hoechst-33342

### **Cell culture**

hES-3 cells were cultured as previously described [1]. Human stem cells were grown on MMC treated fibroblasts' feeder layer. Fibroblasts were plated on gelatine treated dishes. A combination of human and mouse derived fibroblasts were used at a density of approximately 25,000 and  
20 70,000 cells per cm<sup>2</sup> respectively. The fibroblasts were plated up to 48 hours before culture of the stem cells. Mouse fibroblasts only could also support the growth of the stem cells. However, while human fibroblasts could also support stem cells, they created an uneven and unstable feeder layer. Therefore, the human fibroblasts were combined with the mouse fibroblasts  
25 to augment and achieve better support of growth and prevention of differentiation.

The medium that was used for the growth of human stem was DMEM (GIBCO, without sodium pyruvate, with glucose 4500mg/L) supplemented  
30 with 20% FBS (Hyclone, Utah) (2-mercaptoethanol - 0.1mM (GIBCO), Non Essential Amino Acids - NEAA 1% (GIBCO), glutamine 2mM.(GIBCO),

penicillin 50u/ml, and streptomycin 50mg/ml (GIBCO)

For direct observation, hES-3 cells were coated into 12-well plates (3 colonies per well), with or without mouse embryonic feeders (MEFs). The day following the plating, cells were incubated with the different agents in serum free medium containing insulin, transferring and selenium. Media was changed the 2<sup>nd</sup> day and then every 2 days.

For immunostaining, hES-3 cells were coated on chamber slides after mechanical dissociation, in order to obtain a monolayer culture. The day following the plating, cells were incubated with the different agents in a media depleted in serum. Media was changed the 2<sup>nd</sup> day and the cells were fixed 4 days after the first treatment.

For immunoblot analysis, cells were transferred into 24 well plates (8 colonies per well) without MEFs, and 24 hr later, were grown in the absence of serum for 18 hrs.

In some experiments, cells were pre-treated for 1 hr with U0126 (30  $\mu$ M) or for 18 hrs with PTX (100  $\mu$ g/ml).

### ***RT-PCR experiments***

Cells were washed with PBS and hES colonies were removed by treatment with protease. Purified mRNA was extracted from hES cultures using Dynabeads<sup>®</sup> Oligo (dT)<sub>25</sub> (Dyna, Oslo, Norway), according to the supplier's instruction. RT was performed using superscript<sup>™</sup> II Rnase H<sup>-</sup> Reverse Transcriptase (Invitrogen, Life technologies), according to the supplier's protocol. After cooling on ice, the cDNA samples were amplified by PCR with sense and antigens primers (synthesis performed by Sigma Genosys, Castle Hill, Australia) designed for the specific detection of human Edg-1, Edg-2, Edg-3, Edg-4, Edg-5, Edg-6, Edg-7 and Edg-8 DNA target sequences. The primers used for Edg-1, Edg-3, Edg-5, Edg-6 and Edg-8

were previously designed by Hornu *et al.* (2001) [1]. These primer pairs were :

5'-CCACAACGGGAGCAATAACT-3' (sense) and  
5'-GTAAATGATGGGGTTGGTGC-3' (antigens) (expected PCR product: 480  
5 bp) for Edg-1 ;

5'-TCAGGGAGGGCAGTATGTTC-3' (sense) and  
5'-CTGAGCCTTGAAGAGGATGG-3' (antisense) (505 bp) for Edg-3 ;

10 5'-CCAATACCTTGCTCTCTCTGGC-3' (sense) and  
5'-CAGAAGGAGGATGCTGAAGG-3' (antisense) (502 bp) for Edg-5 ;

5'-CGGCTCATTGTTCTGCACTA-3' (sense) and  
5'-GATCATCAGCACCGTCTTCA-3' (antisense) (701 bp) for Edg-6 ;

15 5'-TTCTGATACCAGAGTCCGGG-3' (sense) and  
5'-CAAGGCCTACGTGCTCTTCT-3' (antisense) (460 bp) for Edg-8 .

For Edg-2 and Edg-4, the primer pairs designed by Goetzl *et al.* (1999) were  
20 used:

5'-GCTCCACACACGGATGAGCAACC-3' (sense)  
and 5'-GTGGTCATTGCTGTGAACTCCAGC-3' (antisense) (621 bp) for Edg-  
2,

25 5'-AGCTGCACAGCCGCCTGCCCCGT-3' (sense) and  
5'-TGCTGTGCCATGCCAGACCTTGTC-3' (antisense) (775 bp) for Edg-4.

For Edg-7, the primer pairs designed by Goetzl *et al.* (2000) were used:

5'-CCATAGCAACCTGACCAAAAAGAG-3' (sense) and  
30 5'-TCCTTGTAGGAGTAGATGATGGGG-3' (antisense) (482 bp).

PCR experiments were performed in a mixture (25 µl) containing 0.25  
units of Taq DNA polymerase (Biotech International Ltd, Perth, WA,



Australia) and 2  $\mu$ M of each primer in a buffer including 67 mM Tris-HCl, pH 8.8, 1.5 mM  $MgCl_2$ , 16.6 mM  $[NH_4]_2SO_4$ , 0.45% Triton X-100, 0.25 mM of each dATP, dGTP, dCTP, dTTP. Absence of contaminating genomic DNA was confirmed by control reactions with mRNA that had not been treated with reverse transcriptase. PCR experiments were run with the following steps: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C (Edg-1, Edg-3, Edg-5, Edg-6, Edg-8) or 56°C (Edg-2, Edg-4, Edg-7) for 2 min, extension at 74°C for 2 min, and a final extension at 74°C for 7 min. The specific amplified DNA fragments were purified by electrophoresis on 1.5 % (w/v) agarose gel, stained with ethidium bromide and photographed. The amplicons were purified and sequenced.

### ***Immunofluorescence***

Cells were washed in PBS, fixed with MeOH, and immunostaining was performed, using the specific stem cell marker antibody GCTM-2, and the specific cell proliferation marker PCNA. Cells were then washed and the nucleuses were stained with Hoechst-33342 (1 $\mu$ g/ml). Slides were mounted and then observed by fluorescent microscopy. Cells were then counted in order to determine the ratio of proliferating stem cells within the overall population.

### ***Western blot analysis***

hES3 cells were lysed following removal of the supernatants by addition of a reducing loading buffer (2% SDS, 62.5 mM Tris pH 6.8, 0.1 M DTT, 0.01% bromophenol blue) containing 1 mM sodium orthovanadate. Samples were boiled for 10 min and centrifuged at 13000g for 15 min, and protein lysates (approx. 80  $\mu$ g) were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide, w/v). Proteins were transferred to nitrocellulose (Hybond-ECL, Amersham) and immunoblotting was carried out with rabbit polyclonal anti-active mitogen-activated protein (MAPK) antibodies raised against a dually phosphorylated MAPK peptide (Promega,

Madison, WI, USA). Peroxidase-coupled secondary antibody (Dako) was detected by exposure of autoradiographic films in the presence of a chemiluminescent detection reagent (ECL, Amersham). Stripping of antibodies was achieved by incubating the membrane during 30 min at 50°C  
 5 in a buffer containing 100 mM mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7. The membrane was then reprobing with rabbit polyclonal anti-ERK1/2 antibodies (Promega), and then with peroxidase-coupled secondary antibodies (Dako).

Blots probed with either rabbit polyclonal anti-active p38 (Promega) or  
 10 rabbit polyclonal anti-active JNK (Promega) or mouse polyclonal GCTM-2 antibodies were also performed, using the same procedure as described above.

### ***Protein quantification***

15 hES3 cells were lysed and their quantity was determined by using a colorimetric assay based on the Bradford dye-binding test (Bio-Rad Laboratories, Regents Park, NSW, Australia).

Each set of experiments was performed at least 3 times (*n* refers to number of independent experiments performed on different cell cultures).

20

### **EXAMPLE 3**

The results presented in Figure 7A indicate that hES cells expressed mRNA transcripts for the three S1P receptors : Edg-1, Edg-3 and Edg-5 while these cells do not seem to express mRNA for Edg-6 and Edg-8 (data  
 25 not shown). Moreover, hES cells express mRNA transcripts for each of LPA receptors : Edg-2, Edg-4 and Edg-7 (Figure 7B). The nucleotide sequences of all purified PCR products were analysed and revealed to be identical to the corresponding regions in the human receptor genes.

#### EXAMPLE 4

Applicants next determined whether S1P could modulate the fate of hES cells. When hES cells were grown on MEFs, in a culture media depleted in serum, they spontaneously differentiated. As shown in Figure 8, after 8 days in such conditions, hES cells colonies contained enlarged flattened cells which formed cystic structures (Figure 2A, 2B). Even after 12 days, LPA (up to 50  $\mu$ M) did not seem to affect the growth of the colonies (data not shown). In presence of S1P (10  $\mu$ M, 8 days), the colonies were more compact and less differentiated than in the control condition (Figure 8C). This effect of S1P was more obvious after 12 days of treatment (Figure 8D, 8E). The inhibitory effect of S1P on cell differentiation and the lack of effect of LPA were also observed when hES cells were grown without MEFs, suggesting that S1P did not directly act on the feeder cells (n=3, data not shown).

In order to understand and quantify the effect of S1P on the spontaneous differentiation of hES cells, double immunostaining experiments were carried out. For that purpose, Applicants used two specific antibodies, one as a stem cell marker, GCTM-2, and one for proliferation, PCNA, a marker that is only expressed during the S phase of the cell cycle, in order to determine the ratio of proliferating stem cells (Figure 9). After 4 days in a media without serum, most of the control cells were differentiated (Figures 9A, 9C and 9E), as revealed by the fact that only 16 % of the cells still expressed GCTM-2 (Figure 10A). By contrast, when S1P (10  $\mu$ M) was added to the media, 68 % of the cells were GCTM-2 positive, suggesting that most of the cells remained stem cells (Figures 9B, 9D, 9F and 10B). Within these cell populations, a large part expressed PCNA, suggesting that most of these stem cells still proliferated (Figures 9G and 9H). However, no marked difference in the proliferating rate of hES cells between the control cells and the ones treated with S1P were observed (Figure 10). Altogether, these data suggest that S1P mostly acts on the differentiation of hES cells observed in absence of serum rather than acts on the proliferating state of hES cells.

**EXAMPLE 5**

Because the MAP kinases ERKs have often been implicated in cell proliferation and differentiation, the effects of S1P on the activation of the ERKs were then investigated. After 5 min, S1P stimulated the phosphorylation of ERKs in hES cells (Figure 10), an effect that was totally inhibited in presence of the MEK inhibitor U0126 (30  $\mu$ M) (Figure 10A). S1P stimulated ERKs for at least 60 min and in a concentration dependant manner (Figure 10B, 10C).

These results show clearly that treatment of human ES cells with S1P results in inhibition of spontaneous differentiation. S1P is a major component of serum, and is therefore likely to account for much of the beneficial effect of calf serum in human ES cultures. Although human ES cells express receptors for both S1P and LPA, the latter lysophospholipid is inactive on human ES cells. This suggests that particular members of the Edg receptor family have distinct effects on human ES cell behaviour.

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